(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 846 949 A1

(12) EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

(43) Date of publication:
10.06.1998 Bulletin 1998/24

(21) Application number: **97915725.2**

(22) Date of filing: **15.04.1997**

(51) Int. Cl.[6]: **G01N 33/50**, G01N 33/574

(86) International application number:
PCT/JP97/01300

(87) International publication number:
WO 97/39354 (23.10.1997 Gazette 1997/45)

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **16.04.1996 JP 94050/96**
16.04.1996 JP 94051/96
17.04.1996 JP 95427/96

(71) Applicants:
- **Kishimoto, Tadamitsu**
  Tondabayashi-shi, Osaka 584 (JP)
- **Sugiyama, Haruo**
  Mino-shi, Osaka 562 (JP)

(72) Inventors:
- **SUGIYAMA, Haruo**
  Mino-shi, Osaka 562 (JP)
- **INOUE, Kazushi**
  Ibaraki-shi, Osaka 567 (JP)

(74) Representative:
Kolb, Helga, Dr. Dipl.-Chem. et al
Hoffmann Eitle,
Patent- und Rechtsanwälte,
Arabellastrasse 4
81925 München (DE)

(54) METHOD OF DETECTING SOLID CANCER CELLS AND HISTOLOGICAL HETEROTYPIA AND METHOD OF EXAMINING TISSUE FOR BONE MARROW TRANSPLANTATION AND PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

(57) The invention provides a method of detecting solid cancer cells which comprises determining the level of expression of WT1 gene in a test tissue, a method of detecting the atypia of a tissue which comprises determining the level of expression of WT1 gene in the tissue, and a method of testing a graft material tissue for bone marrow or peripheral blood stem cell transplantation which comprises determining the level of expression of WT1 gene in a $CD34^-$ cell fraction of the tissue to detect leukemic cells and solid cancer cells in the tissue.

EP 0 846 949 A1

marrow or peripheral blood stem cells, but there has not been available an established technology for testing whether leukemic cells are included among myelocytes or peripheral blood stem cells.

It is, therefore, an object of the present invention to provide a technology for quantitating tumor cells which is of value for the diagnosis of solid cancers, more particularly a quantitative test utilizing a clinical marker correlated with solid cancers.

Another object of the present invention is to provide a technology for detecting atypia utilizing a novel marker by which the risk of acute leukemic transformation, for instance, can be predicted with greater accuracy taking into consideration the course and prognosis of MDS in particular.

It is a still another object of the present invention to provide a novel technology which permits detection of leukemic cells in the tissue for use in said bone marrow transplantation or peripheral blood stem cell transplantation.

DISCLOSURE OF THE INVENTION

As the result of their intensive research directed to the above-mentioned objects, the inventors of the present invention discovered for the first time that the WT1 gene, which is a well-known clinical marker of leukemia, shows a significantly high level of expression in solid cancers which are unrelated to leukemia. Accordingly they found that this gene can be effectively utilized in the detection of solid cancer cells.

The inventors further found that the level of WT1 gene expression is also high in MDS and other variants which are clearly differentiated from leukemia and that the expression level of the WT1 gene in such a variant can be an indicator of acute leukemic transformation.

Furthermore, the inventors of the present invention found that although the expression level of the WT1 gene in the $CD34^+$ cell fraction of a graft material cannot be an indicator of contamination with leukemic cells, the level of WT1 gene expression in the $CD34^-$ cell fraction can be a good marker of contamination with leukemic cells and solid cancer cells and that determination of this level enables the detection of leukemic cells and solid cancer cells. The present invention has been developed on the basis of the above findings.

The present invention provides a method for detecting cancer cells which comprises determining the level of expression of the WT1 gene in a tissue to be tested to thereby detect solid cancer cells.

The present invention also provides a method for detecting the atypia of a tissue to be tested which comprises determining the level of expression of the WT1 gene in said tissue.

Particularly, this method for detection of atypia is preferably applied when the tissue to be tested is one presenting with myelodysplastic syndrome (preleukemia) or when the determination of the expression level of the WT1 gene is made on its transcript.

The term "atypia" is used herein to mean a morphological variation from the normal tissue or cell and its degree is generally parallel to the malignancy of a tumor [Saishin Igaku Dai-Jiten (Contemporary and Comprehensive Medical Encyclopedia), June 15, 1987, Ishiyaku Shuppan].

The method for detecting atypia according to the invention is essentially predicated on the use of the level of WT1 gene expression as a test for atypia, that is to say the use of said expression level as an indicator of acute leukemic transformation in MDS and other diseases mentioned above. As such, the method has a great clinical significance for assessment of the prognosis or course of such diseases inclusive of MDS.

The method for detecting atypia according to the invention has the following additional clinical significance. Thus, taking a patient with MDS as an example, when the WT1 value determined by the method of the invention increases gradually from a low level, it can be considered that the patient has a high risk for acute leukemia, thus indicating an early allogenic bone marrow transplantation or allogenic peripheral blood stem cell transplantation.

Furthermore, the method for detecting atypia according to the invention can be used for diagnosing whether atypical cells have emerged in blood in healthy humans, victims of radiation hazards (the personnel employed in an atomic power plant, workers handling radiations, patients on radiation therapy, etc.), persons who received anticancer therapy for breast cancer, and patients presenting with leukemoid symptoms induced by infections.

In addition, the present invention provides a method for testing a tissue for use as a graft material in bone marrow transplantation or peripheral blood stem cell transplantation, which comprises determining the level of WT1 expression in the $CD34^-$ cell fraction of a graft material for transplantation to thereby detect leukemic cells and solid cancer cells in said material.

The abbreviations used for amino acids, peptides, nucleotide or base sequences, and nucleic acids in this specification are conforming to those adopted by IUPAC-IUB or the "Guideline for the Drafting of Specifications Including Base Sequences or Amino Acid Sequences" (edited by the Japanese Patent Office) and those in routine use in the art.

The WT1 gene which is determined by the technology of the present invention is known as such and its base sequence is shown in the literature [e.g. Cell, 60, 509 (1990); Nature, 343, 774 (1990)].

What counts in the technology of the invention is to determine the expression level of said WT1 gene in a test tissue or a $CD34^-$ cell fraction thereof and this is the most outstanding feature shared by the various methods disclosed herein.

as well and, together with the example given hereinafter, can be consulted as references.

In carrying out the detection technology of the present invention, it is advantageous to use a solid cancer and atypia detection kit containing suitable reagents for determining the expression level of the WT1 gene as active ingredients.

The detection kit should contain specific reagents tailored to the specific method of determining the expression level of the WT1 gene as essential components. Such specific reagents are appropriately chosen and established according to the particular detection protocol adopted and are defined as reagents necessary for the means for specific detection of the target according to the invention, such as anti-WT1 protein antibody, WT1 gene transcript detection probe, and/or the corresponding primer set, among others. Furthermore, although not considered to be essential components of such a detection kit, reagents for PCR may also be included in the kit just like the reagents for hybridization, for instance. The present invention, thus, provides a kit for the detection of solid cancer cells and atypia and a kit for cancer diagnosis.

The method for testing tissues for bone marrow transplantation or peripheral blood stem cell transplantation is now described in detail. This test method comprises determining the level of WT1 expression in a $CD34^-$ cell fraction available upon elimination of $CD34^+$ cells from a graft material.

This determination of the expression level of the WT1 gene can be carried out by using a variety of techniques just as mentioned for the method for detecting solid cancer cells and atypia which has been described hereinbefore.

The sample that can be used for this test is the $CD34^-$ cell fraction available upon elimination of $CD34^+$ cells from any tissue for use as a graft material by a well-known suitable technique. As examples, such fractions of peripheral blood stem cells, bone marrow fluid, etc. can be mentioned. Such a $CD34^-$ cell fraction can be isolated by the routine procedure, typically column chromatography using a CD34 column or a substance having an affinity for CD34 cells, such as FACS, or a magnetic cell separator. The column that can be typically used for this procedure includes Ceparate (manufactured by Cell-Pro), Isolex 300 and 50 (Baxter), and MaCS (Amzene), among others.

The separation procedure using a magnetic cell separator is now described in detail. First, the mobilized peripheral blood available upon elimination of platelets etc. by centrifugation or the like or the bone marrow fluid available upon elimination of mononuclear cells by means of a Ficoll pack or the like and subsequent centrifugation is reacted with anti-CD34 monoclonal antibody for sensitization and, then, reacted with Dynabeads to which a secondary antibody has been conjugated to cause rosetting. This rosette (positive cell-antibody-Dynabead complex) is magnetically separated from non-rosetting cells (negative cells). From the rosette thus obtained, positive cells can be harvested by enzymatic treatment with chymopapain.

It has been found that this method of determining the level of WT1 gene expression in the above sample, that is a transcript or translation product of the WT1 gene, provides a value well correlated with the available number of leukemic cells or solid cancer cells and that when this measured value is not over the 10^{-4} level, the sample can be assessed to contain substantially no leukemic or solid cancer cells. Therefore, in accordance with this invention, the presence of leukemic cells and solid cancer cells in a graft material for transplantation can be tested and, hence, the safety of the graft material can be assured.

In the above test for graft materials in accordance with the invention, reagents similar to those already mentioned for the detection-diagnostic kit for solid cancer cells and atypia can be utilized, and the present invention further provides such a test kit for graft materials.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagrammatic representation of the data generated by determining the level of WT1 gene expression in various cancer cells in accordance with Example 1.

Fig. 2 is a diagrammatic representation of the plots obtained by determining the level of WT1 gene expression in various patients in accordance with Example 2.

Fig. 3 is a diagrammatic representation of the data generated by determining the level of WT1 gene expression in various $CD34^-$ cell fractions in accordance with Example 3.

BEST MODE FOR PRACTICING THE INVENTION

The following examples are further illustrative of the present invention in detail.

Example 1

Detection of solid cancer cells

As solid cancer cells, the following cell lines derived from various tissues were used. The JCRB number of each cell line is one assigned by Japanese Cancer Research Bank (JCRB).

PCR was performed on a DNA thermal cycler (Perkin Elmer-Cetus) in a round of cycles of denaturation at 94°C x 1 min., primer annealing at 64°C x 1 min. (β actin: 60°C x 1 min.), and chain elongation at 72°C x 2 min. to provide a PCR product (first round PCR).

When the densitometer unit (described below) of this PCR product was less than 500, a second round PCR with the nested inner primer set was carried out using a 2.5 µl aliquot of the first round PCR product.

The PCR product thus obtained was assayed in accordance with the protocol given in the literature [J. Immunol., 147, 4307 (1991)], as follows.

Thus, the PCR product from 20 ng of total RNA was cloned on 1.3% agarose gel containing 0.05 µg/ml of ethidium bromide and photographed using polaroid film (Polaroid 665 film, Polaroid Corp.).

The negative film was developed at 25°C for 5 minutes and scanned with a densitomer (CS-9000, Shimadzu) to find the "densitomer unit".

The result for the PCR product obtained in the absence of RNA under otherwise the same conditions was used as negative control.

The primers used in the above procedures are shown in Table 1.

Table 1

First round PCR primers	Base sequences
Outer sense primer	5'-GGCATCTGAGACCAGTGAGAA-3'
Outer antisense primer	5'-GAGAGTCAGACTTGAAAGCAGT-3'
Second round PCR primer	Base sequences
Inner sense primer	5'-GCTGTCCCACTTACAGATGCA-3'
Inner antisense primer	5'-TCAAAGCGCCAGCTGGAGTTT-3'

As the primers for β actin used as an internal control, those described in the literature [Proc. Natl. Acad. Sci. USA., 82, 6133, (1985)] were used. The respective primers were invariably synthesized by the conventional chemical method.

To standardize the amount of RNA for RT-PCR and the difference in RNA decomposition of samples, the result (densitomer unit) with the WT1 gene was divided by the result with β actin and the value was used as the level of WT1 expression.

Furthermore, with the level of WT1 gene expression in the cell line K562 known to have the WT1 gene expressed [Lozzio, C. B. and Lozzio, B. B., Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome, Blood, 45, 321-334 (1995)] as determined by the above procedure being taken as the reference (1.00), the levels of WT1 gene expression in various test cells were calculated as relative ratios.

The results are presented in Fig. 1.

In Fig. 1, the ordinate represents the relative ratios of WT1 gene expression in various cell lines with the level of WT1 gene expression in the cell line K562 (K562 in the table) being taken as 1.00, while the abscissa represents test cells (gastric cancer, colon cancer, lung cancer, breast cancer, germ cell tumor, ovary cancer, and thyroid cancer cells, represented by the numbers assigned hereinbefore).

It is apparent from Fig. 1 that WT1 has been highly expressed in all the gastric cancer, colon cancer, lung cancer, breast cancer, germ cell tumor, ovary cancer, and thyroid cancer cells, indicating that WT1 can be used as a tumor marker for such solid cancer cells. It is accordingly clear that a clinically valuable, novel detection and assay technology for solid cancers has been established.

Example 2

Detection of MDS

(1) Determination of the level of WT1 gene expression

The level of WT1 gene expression was determined in accordance with the protocol described in the literature [Blood, 84(9): 3071, 1994], as follows.

Thus, total RNA was extracted from each sample by the routine method [acid-guanidine-phenol-chloroform method, Anal. Biochem., 162, 156 (1987)], dissolved in diethyl pyrocarbonate-treated water, and quantitated optically at 260 nm.

Thus, in accordance with the technology of the invention, not only the detection of atypia is made feasible but the risk of leukemic transformation in MDS patients can be predicted by serial determination of the level of WT1 gene expression.

Example 3

Testing graft materials

As graft materials for peripheral blood stem cell transplantation, peripheral blood stem cells were isolated from various patients to whom granulocyte colony stimulating factor (G-CSF; "Gran", product of Kirin-Sankyo, or "Neutrogin", prosuct of Chugain) had been administered ahead of time (autologous peripheral blood stem cell transplantation: 2 μg/kg body weight x 3~7 days; allogenic peripheral blood stem cell transplantation: 10 μg/kg body weight x 7 days) had been administered ahead of time. From the isolated peripheral blood stem cells, $CD34^-$ cells were separated and recovered using a cell separation system (Isolex 50, product of Baxter) [e.g. J. Hematotherapy, 4, 531-438 (1995)].

This procedure was carried out in accordance with the manufacturer's manual. The cells were first reacted with anti-CD34 monoclonal antibody and then reacted with Dynabeads conjugated with a secondary antibody for rosetting. This rosette (positive cell-antibody-Dynabeads complex) is separated from non-rosetting cells (negative cells). From this rosette, positive cells can be recovered by enzymatic treatment with chymopapain.

The expression level of the WT1 gene in the $CD34^-$ fractions from said patients were determined in accordance with the protocol described in the literature [Blood, 84(9), 3071 (1994)], as follows.

Thus, total RNA was extracted from each fraction by the routine method [e.g. acid-guanidine-phenol-chloroform method; Anal. Biochem., 162, 156 (1987)], dissolved in diethyl pyrocarbonate-treated water, and quantitated optically at 260 nm.

Thus, 15.5 μl of diethyl pyrocarbonate-treated water containing 1 μg of total RNA was heated at 65°C for 5 minutes and mixed with 14.5 μl of RT buffer (50 mmol/l Tris-HCl (pH 8.3), 70 mmol/l KCl, 3 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol) containing 600 U reverse transcriptase (Moloney murine leukemia virus reverse transcriptase, GIBCO-BRL), 500 mmol/l each deoxynucleotide triphosphate (dNTP, Pharmacia), 750 ng oligo dT primer set, and 40 U RNase inhibitor (Boehringer Mannheim). This mixture was incubated at 37°C for 90 minutes, heated at 70°C for 20 minutes, and stored at -20°C until use.

PCR was performed on a DNA thermal cycler (Perkin Elmer-Cetus) in a round of cycles of denaturation at 94°C x 1 min., primer annealing at 64°C x 1 min. (β actin: 60°C x 1 min.), and chain elongation at 72°C x 1 min. to provide a PCR product (first round PCR).

When the densitometer unit (described below) of this PCR product was less than 500, a second round PCR with the nested inner primer set was carried out using a 2.5 μl aliquot of the first-round PCR product.

The PCR product thus obtained was assayed in accordance with the protocol given in the literature [J. Immunol., 147, 4307 (1991)], as follows.

Thus, the PCR product from 20 ng of total RNA was cloned on 1.3% agarose gel containing 0.05 μg/ml of ethidium bromide and photographed using polaroid film (Polaroid 665 film, Polaroid Corp.). The negative film was developed at 25°C for 5 minutes and scanned with a densitomer (CS-9000, Shimadzu) to find the "densitomer unit". The result for the PCR product obtained in the absence of RNA under otherwise the same conditions was used as negative control. The primers used in the above procedures are shown in Table 3.

Table 3

First round PCR primers	Base sequences
Outer sense primer	5'-GGCATCTGAGACCAGTGAGAA-3'
Outer antisense primer	5'-GAGAGTCAGACTTGAAAGCAGT-3'
Second round PCR primer	Base sequences
Inner sense primer	5'-GCTGTCCCACTTACAGATGCA-3'
Inner antisense primer	5'-TCAAAGCGCCAGCTGGAGTTT-3'

As the primers for β actin used as an internal control, those described in the literature [Proc. Natl. Acad. Sci. USA., 82, 6133, (1985)] were used. The respective primers were invariably synthesized by the conventional chemical method.

To standardize the amount of RNA for RT-PCR and the difference in RNA decomposition of samples, the result

Claims

1. A method for detecting cancer cells which comprises determining the level of expression of the WT1 gene in a test tissue to detect solid cancer cells.

2. The detection method according to Claim 1 wherein the level of expression of the WT1 gene is determined on a transcript of said gene.

3. The detection method according to Claim 1 wherein the solid cancer cells are selected from among gastric cancer, colon cancer, lung cancer, breast cancer, germ cell tumor, ovary cancer, and thyroid cancer cells.

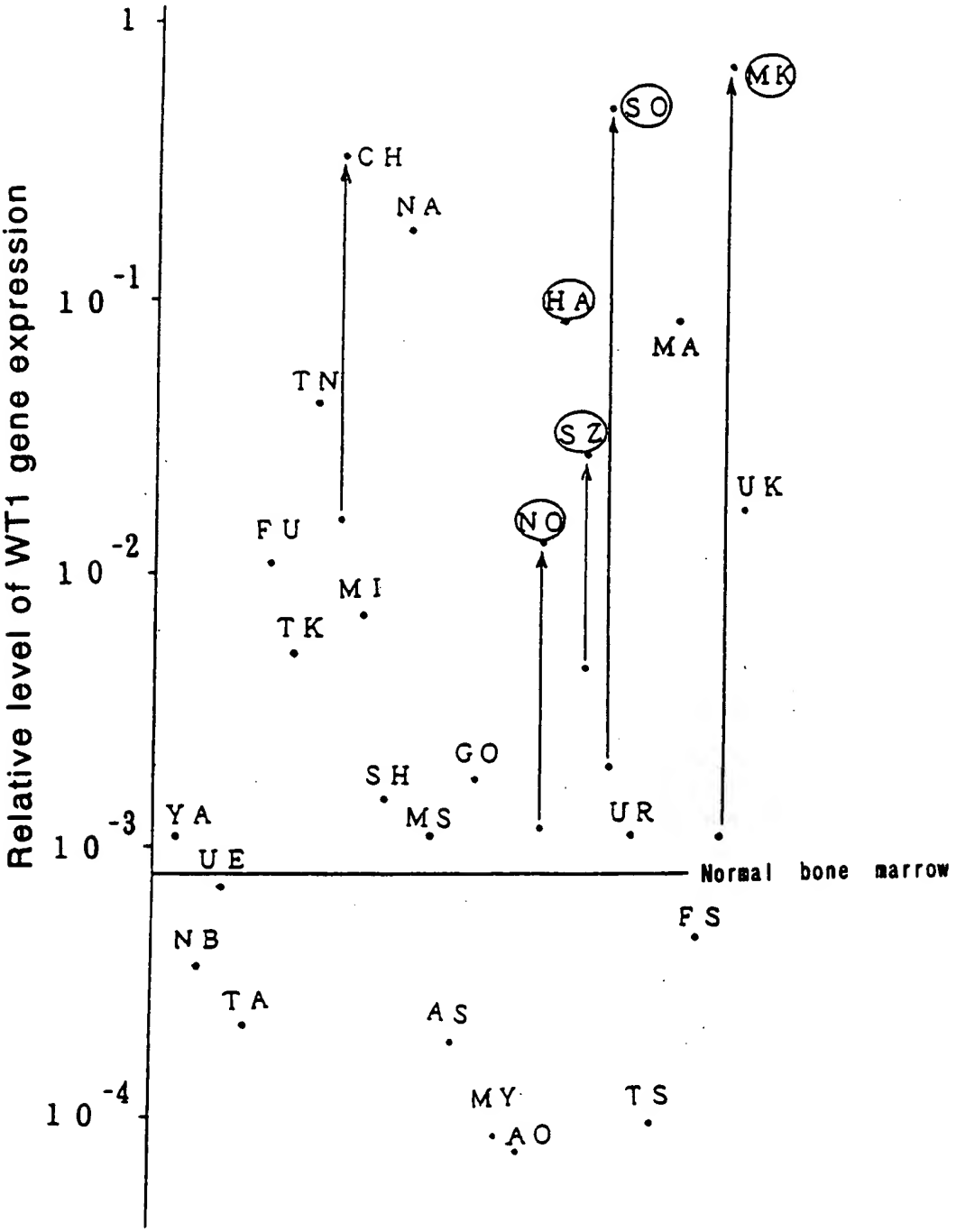
4. A method for detecting atypia which comprises determining the level of expression of the WT1 gene in a test tissue to thereby detect atypia.

5. The detection method according to Claim 4 wherein the test tissue is one from a patient with myelodysplastic syndrome.

6. The detection method according to Claim 4 wherein the level of expression of the WT1 gene is determined on a transcript of said gene.

7. A method for testing a graft material tissue for bone marrow transplantation or peripheral blood stem cell transplantation which comprises determining the level of expression of the WT1 gene in a $CD34^-$ cell fraction of said graft material tissue to detect leukemic and solid cancer cells in the tissue.

FIG. 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP97/01300

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl^6 G01N33/50, 33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int. Cl^6 G01N33/50, 33/574

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1940 - 1997
Kokai Jitsuyo Shinan Koho 1971 - 1997
Toroku Jitsuyo Shinan Koho 1994 - 1997

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS, "WT1*CANCER"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	W. Bruening et al., "Analysis of the 11P13 Wilms' Tumor Suppressor Gene (WT1) in Ovarian Tumors" Cancer Investigation, Vol. 11, No. 4, (1993), p. 393-399	1 - 7
Y	W.L. Gerald et al., "Expression of the 11P13 Wilms' Tumor Gene, WT1, Correlates with Histologic Category of Wilms' Tumor" American Journal of Pathology, Vol. 140, No. 5, (1992), p. 1031-1037	1 - 7
A	K. Inoue et al., "WT1 as a New Prognostic Factor and a New Marker for the Detection of Minimal Residual Disease in Acute Leukemia" Blood, Vol. 84, No. 9, (1994), p. 3071-3079	1 - 7

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
July 29, 1997 (29. 07. 97)	August 12, 1997 (12. 08. 97)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)